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Synthesis and *in vitro* behavior of β-TCP zirconia/polymeric biocomposites for bio-applications

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Abstract Novel biocomposites were fabricated by impregnating β-tricalcium phosphate (β-TCP)/zirconia particles into the polymers matrix. The composite materials were characterized using thermo-gravimetric analysis (TGA), X-ray diffraction (XRD), Fourier Transform Infrared (FT-IR) analyzes and Scanning Electron Microscopy (SEM). The results confirmed the conversion of hydroxyapatite (HA) to β-TCP at a sintering temperature of 1150 °C with or without zirconia powder. The *in vitro* behavior was assessed via measurement of calcium and phosphorus ions in SBF (simulated body fluid). FT-IR and SEM of the composites were performed pre and post immersion in SBF. The results prove that the bone like apatite layer formation was enhanced on the β-TCP-Z20/polymeric composite surface more than that on the β-TCP-Z10/polymeric composite. Therefore, the data confirmed that zirconia plays an important role in the enhancement of the apatite formation. The conclusions proved that the β-TCP-Z20/polymeric biocomposites, containing 20% of zirconia, are promising for bone remodeling applications.

1. Introduction

β-TCP ceramics have been commercialized as bioresorbable synthetic bone substitutes and are used in orthopedic and dental applications, including augmentation of the alveolar ridge, sinus reconstruction and bone substitutes and are used in orthopedic and dental applications, including augmentation of the alveolar ridge, sinus reconstruction and general bone reconstruction following injury or disease. β-TCP bioceramics are widely used as bone replacements in the field of oral and plastic surgery [12]. The clinical applications are being limited because of the brittleness of calcium phosphate ceramics and difficulty of shaping [23]. Zirconia can be a substitute to alu-

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mina for the ceramic ball heads of hip prostheses, because of higher fracture toughness. It also exhibits the best mechanical properties of oxide ceramics; this is the consequence of phase transformation toughening, which increases its crack propagation resistance [5]. The incorporation of ZrO_2 into a bioactive matrix can be useful for improving the mechanical properties without substantial effect on biocompatibility [20]. The addition of zirconia improved the resorbable nature of HA by producing an adequate β-TCP phase which is characterized by high resorbability. The prepared β-calcium phosphate ceramics definitely have better bioresorbability than HA and it may have excellent mechanical strength due to zirconia reinforcement [17].

Chitosan is biodegradable, biocompatible and nontoxic [15]. The surface of chitosan is hydrophilic, which facilitates cell adhesion, proliferation, and differentiation [14]. The incorporation of calcium phosphate ceramic into chitosan–gelatin polymeric matrix has been recently shown to increase its osteoconductivity, as well as improving stem cells adhesion and differentiation [25]. Gelatin is a denatured collagen and is commercially available as a biodegradable polymer. Other advantages of gelatin include the easiness of chemical modification and its use with different charges. In addition, biodegradable polymers, such as chitosan [25] and gelatin [21] polymers can be used as binders for ceramics to reduce their brittleness [8]. Moreover, the additions of ceramic granules reinforce and stiffen the polymeric matrix [3].

The present study aimed at the firing of hydroxyapatite with or without zirconia powder at temperature 1150 °C for 2 h as soaking time to produce β-TCP–zirconia and β-TCP powders. The prepared β-TCP/ and β-TCP–zirconia/polymeric composites were characterized and immersed in SBF solution to confirm the formation of apatite layer on their surfaces.

2. Materials and methods

2.1. Materials

Hydroxyapatite powder (Merk), zirconium oxide powder (Laboratory Rasyan, S.D., Fine chemical Ltd.), chitosan powder (Aldrich), gelatin powder (BDH laboratory supplies Poole, BH15 1TD, England) and 2-hydroxyethylmethacrylate monomer (HEMA) (Aldrich) were used in this work. The chemical initiators, cerric ammonium nitrate (CAN) (BDH, UK), sodium hydrogen sulfite and ammonium persulfate were used to graft HEMA monomer onto chitosan and gelatin polymers.

2.2. Methods

2.2.1. Preparation of fillers powder

Zirconia powder of 10% and 20% weight concentration was well mixed with HA powder. HA, HA/10% zirconia and HA/20% zirconia powders were pressed, molded and fired at 1150 °C for 2 h as soaking time. After the heating process, the samples were ground and sieved up to 100 μm. Firing of HA alone leads to the decomposition to β-TCP as given in Eq. (1), while firing of HA with ZrO, produce β-TCP and calcium zirconium oxide as in Eq. (2) (12).

The powder samples were denoted as β-TCP, β-TCP-Z10 and β-TCP-Z20powders.

$$Ca_{10}(PO_4)_6OH_2 - - - - - - - - - - - - - - - 3Ca(PO_4)_2 + CaO + H_2O \quad (1)$$

$$Ca_{10}(PO_4)_6OH_2 + ZrO2 - - - - - - - - - - - -3Ca(PO_4)_2 + CaZrO_3 + H_2O \quad (2)$$

2.2.2. Preparation of polymeric matrix

The polymeric matrix was prepared according to Mohamed and Mostafa, [16]. In explanation, chitosan polymer (0.05 g) was dissolved in 4.0 ml of 3% acetic acid solution then 1.0 ml of HEMA monomer and 0.05 g of cerric ammonium nitrate (CAN) was used as initiator, in order to achieve the grafting of HEMA monomer onto chitosan polymer. The mixture was well mixed and kept in a water bath at 40 °C for 2 h to obtain a homogenous copolymer mixture, which is denoted as solution (1). Meanwhile, gelatin (0.05 g) was dissolved in 4 ml distilled water then 1.0 ml of HEMA monomer and 0.05 of initiator mixture (sodium hydrogen sulfite and ammonium per sulfate) were added, well mixed and incubated in a water bath at 40 °C for 2 h to obtain a homogenous copolymer mixture which is denoted as solution (2). The solutions (1) and (2) were well mixed and incubated at 40 °C for 1 h in a water bath. The copolymer mixture was left under stirring for overnight at room temperature, then washed with ethanol for 2 h to remove the remaining homopolymer. The copolymer mixture was filtered, collected and dried at 60 °C overnight till constant weight. The grafting percentage ($G\%$) of HEMA monomer onto the two polymers for forming the copolymeric or polymeric matrix was calculated according to the following equation [18]:

$$G\% = [(W_o - W)/W_o] \times 100$$

where (W_o) is weight in g of the original chitosan and gelatin, and (W) is weight in g of the grafted chitosan and gelatin (copolymer).

2.2.3. Preparation of β-TCP/polymeric composites

The above steps for the preparation of polymeric matrix were repeated. Then, the fixed weight (1.75 g) of each filler powder was dispersed into the polymeric matrix 30 min before the end of copolymerization process, well mixed and incubated for 30 min under the same above conditions. The filler/polymeric composites were left overnight at room temperature, then filtrated, washed and dried at 60 °C overnight. The prepared composites were denoted as β-TCP/polymeric composite/, β-TCP-Z10 polymeric composite/and β-TCP-Z20/polymeric composite. The grafting percentage of HEMA onto both polymers in the presence of the filler powder (filler/polymeric composites) was calculated according to the following equation:

$$G\% = [(W_t - W_o)/W_o] \times 100$$

where W_t is weight in g of the grafted copolymer containing filler powder.

W_o is weight in g of the chitosan and gelatin polymers powders.

2.3. Characterization of the as prepared samples

The weight of the prepared materials were evaluated by thermo gravimetric analysis (TGA) using a Perkin-Elmer, 7 series thermal analyzer at a heating rate of 10 °C/min over the temperature range of 50–1000 °C. The phase analysis of the samples was examined by X-ray diffractometer (Diana Corporation, USA) equipped with Co Kα radiation, $X = 1.79026$ Å

with Fe filter. The FT-IR spectra were measured using KBr pellets made from a mixture of powder for each sample and were assessed from 400 to 4000 cm^{-1} using a Nexus 670, Nicloet FT-IR spectrometer, USA. SEM micrographs of the composites were also studied using SEM Model Philips XL 30, with accelerating voltage 30 kV, magnification from 10× up to 400,000×.

2.4. Properties of the composites samples

2.4.1. Water absorption (WA%)

Water absorption (WA%) studies are of great importance for a biodegradable material. All the specimens were weighed before being immersed in distilled water. The samples were carefully removed from the medium and immediately weighed for determination of the wet weight as function of the immersion time [6]. Water absorption of the copolymer is given according to the following equation:

$$WA\% = [(W_f - W_i)/W_i] \times 100$$

where W_i is the initial weight of the sample, and W_f is the sample weight after immersion. Each experiment was repeated three times and the average value was taken to ensure the results.

2.4.2. In vitro behavior

The samples were soaked in SBF, proposed by Kokubo et al. [9] at body temperature (37 °C) and pH = 7.4 for several periods. In briefly, SBF contains ions similar to those in human blood plasma and was prepared by dissolving the following reagent grades: NaCl (8.035 g), $NaHCO_3$ (0.355 g), KCl (0.225 g), $K_2HPO_4.3H2O$ (0.231 g), $MgCl_2.6H2O$ (0.311 g), $CaCl_2$ (0.292 g) and Na_2SO4 (0.072 g) in ion exchanged distilled water. The solution was buffered at pH 7.4 with Tris (hydroxymethylaminomethane) $[(CH_2OH)_3CNH_2)]$ (6.118 g) and 1 M hydrochloric acid (HCl) (39 ml). After immersion, the solutions were analyzed by the spectrophotometer (UV-2401PC,UV–VIS recording spectrophotometer, Shimadzu, Japan) using biochemical kits (Techo Diagonstic, USA) to detect the total calcium ions (Ca^{2+}) at λ = 570 nm and phosphorus ions (P) concentration at λ = 675 nm. The immersed specimens were removed from the SBF, then abundantly rinsed using de-ionized water and dried for FT-IR and SEM investigations to show the formation of bone-like apatite layer on their composite surfaces.

3. Results and discussion

3.1. The grafting (G%)

Table 1 shows the G% of the prepared copolymer and the composite samples. The grafting values were high for β-TCP/polymeric composite, β-TCP-Z10 polymeric composite, β-TCP-Z20 polymeric composite (1137%), (1102%) and (1128%), respectively, compared to the polymeric matrix (503%) due to the presence of the filler powder which reinforces the composite forming the integrated composite.

3.2. Thermal analysis (TGA)

The TGA results show that the destruction of the copolymer started at 235 °C and recorded a total weight loss (97.83%) as a result of the release of all NH_3, CO_2 gases and H_2O molecules [2] (Fig. 1a). The weight loss of β-TCP filler recorded 0.32 due to release of the absorbed water onto the surface of the particles. The destruction of β-TCP/, β-TCP-Z10/ and β-TCP-Z20/polymeric composites started at 218, 197 and 218 °C, respectively (Fig. 1c–e and Table 1). Therefore, the grafting (%) and TGA data confirmed the presence of the same amounts of attached polymer for the three β-TCP/polymeric composites, nevertheless the content of zirconia powder.

3.3. Phase analysis

The XRD patterns indicate that HA was converted to β-TCP at 1150 °C. The prepared powder had three main peaks at dA = 2.79, 3.44 and 2.71 (Card No. 74-0565) proving the formation of. HA material (Fig. 2a). The HA powder sintered at 1150 °C had three main peaks at dA = 2.88, 3.20 and 2.60 (Card 13 No. 70-2065) denoting the formation of β-TCP compound (Fig. 2c). This coincided with Raynaud et al. who reported that HA is transformed to β-TCP at 1170 °C [19]. The peaks of β-TCP appeared in β-TCP-Z10 or β-TCP-Z20 powders and their intensities were reduced compared to the original β-TCP powder proving some interaction (Fig. 2d,e). Also, it is noted that the peaks of zirconia (Card No. 83-0944) disappeared in β-TCP-Z10 and β-TCP-Z20 composite powders. A new peak is appeared at dA = 2.95 in β-TCP-Z10 composite powder and its intensity was enhanced with the increase of zirconia in β-TCP-Z20 composite powder. This peak belongs to calcium zirconium oxide (Card No. 26-341) proving interaction between HA and zirconia at 1150 °C [11] as exchange of Ca^{2+} and Zr^{++} ions between HA and zirconia (Fig. 2). The XRD patterns indicate that the peaks of β-TCP in the β-TCP-Z/polymeric composite have slightly lower intensities compared with the β-TCP sample proving the coating effect by a polymeric layer The peaks of the copolymer in all composites disappeared denoting the effect of filler on the sites of polymer bands or the amorphous nature of the copolymer (Fig. 3).

3.4. FT-IR analysis

The bands of chitosan and gelatin polymers appeared as follow, OH band at 3340 cm^{-1}, amide I at 1650 cm^{-1}, C═O at 1624 cm^{-1}, C—O at 1050 cm^{-1} and CH band at 2850 and 1460 cm^{-1} [4].

Table 1 The grafting and weight loss % of the copolymer and their composites.

Sample	Copolymer matrix	β-TCP/polymeric comp.	β-TCP-Z10/polymeric comp.	β-TCP-Z20/polymeric comp.
Grafting (%)	503	1137	1102	1128
Weight loss (%)	97.83	48.314	47.869	48.10

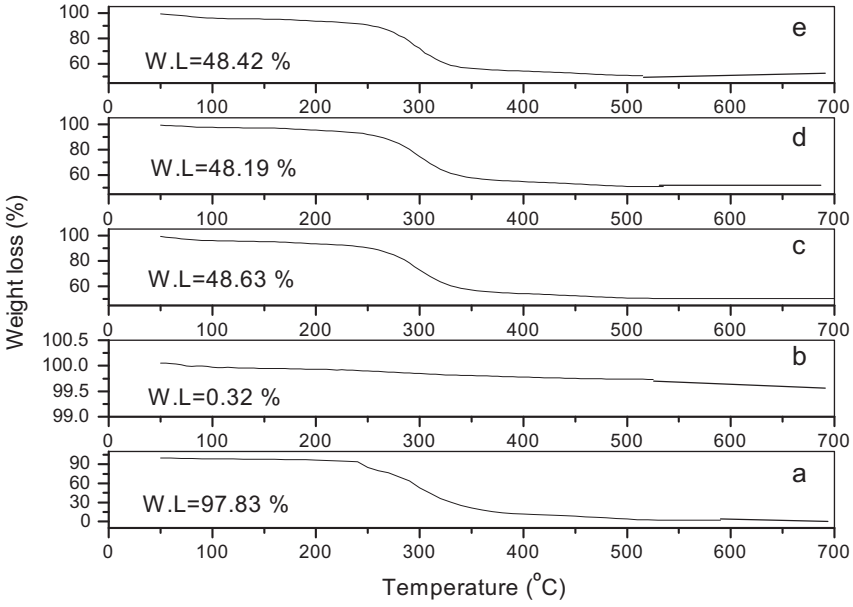

Figure 1 TGA of (a) copolymer, (b) β-TCP powder, (c) β-TCP/, (d) β-TCP-Z10/ and (e) β-TCP-Z20/polymeric composites.

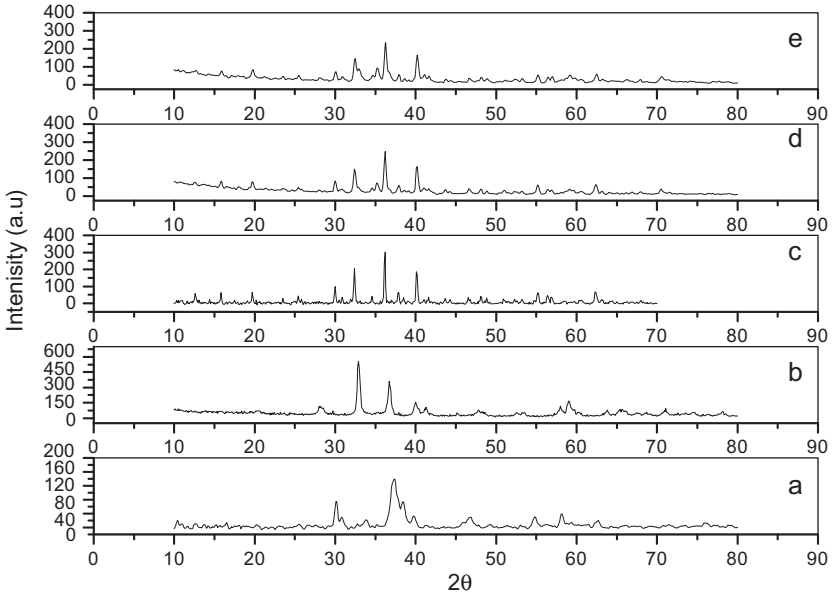

Figure 2 XRD of (a) HA powder, (b) zirconia powder, (c) β-TCP, (d) TCP-Z10 and (e) TCP-Z20 filler powders.

Also, the bands of HEMA were assigned to OH at 3340 cm^{-1}, CH and/or OH at 2940 and 2850 cm^{-1}, C═O at 1720 cm^{-1}, C─O at 1160 cm^{-1} and CCO at 1280 cm^{-1} [13]. For the grafted copolymer, the intensities of all above bands were reduced with some shift compared to those bands in chitosan, gelatin and HEMA proving chemical interaction between both polymers and HEMA monomer (Fig. 4). The phosphate bands at 1089, 1045 and 961, 601 and 571 cm^{-1} had reduced intensities compared to the original bands in the fillers powder proving the coating effect. Also, the bands of the copolymer in the composites especially β-TCP-Z10/polymeric composite were markedly reduced compared to original copolymer (Fig. 5).

3.5. Water absorption (WA%)

The WA% of the copolymer recorded high values compared to the three composites proving high affinity of the polymeric matrix to water molecules (Fig. 6). The results proved the stability of these composites in the media due to the presence of temporary filler barriers which prevent water permeability into the co-polymeric. This result is coincided with Tharanathan et al. [22] and Abeer et al. [1]. It was noted that the blending of the material powder with a hydrophilic polymeric matrix reduced the water absorption compared to the polymeric matrix alone.

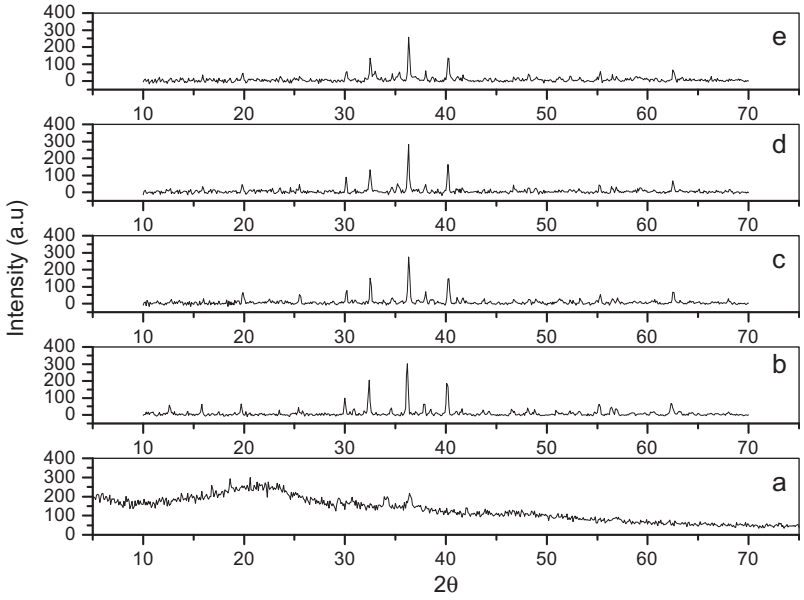

Figure 3 XRD patterns of (a) copolymer, (b) β-TCP powder, (c) β-TCP, (d) β-TCP-Z10 and (e) β-TCP-Z20 polymeric composites.

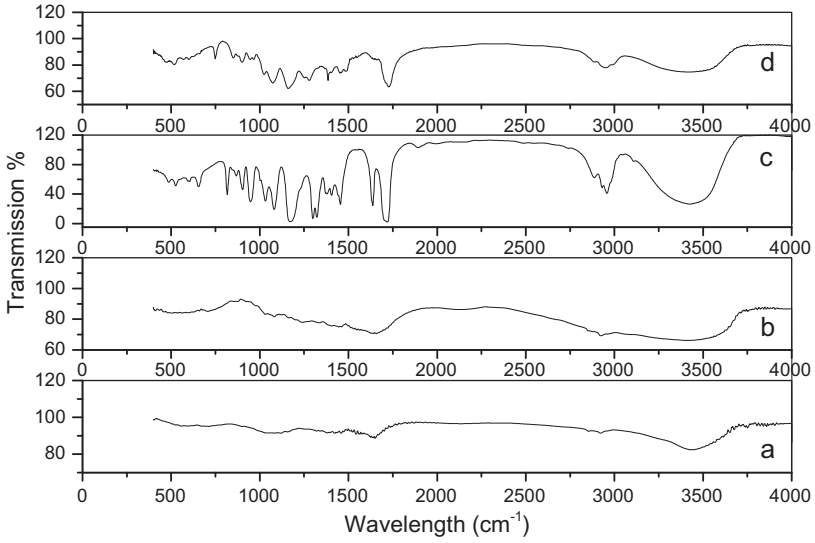

Figure 4 FT-IR of (a) chitosan, (b)gelatin, (c) HEMA and (d) copolymer matrix.

3.6. The in vitro behavior

3.6.1. In vitro test

3.6.1.1. Calcium ions (Ca^{++}). The concentration of Ca^{++} ions recorded low values for the three composites and the copolymer compared to the control proving deposition of Ca^{++} ions onto their surfaces. This result proves the interaction between the amino groups of both polymers and the Ca^{++} ions in SBF via hydrogen bonding (Fig. 7). In this domain, Feng et al. reported that the enhancement of calcium ions adsorption on chitosan–gelatin polymer network improved the osteogenic differentiation [7].

3.6.1.2. Phosphorus ions (P). The concentration of P ions post-immersion recorded lower values for the copolymer compared to the control and the three composites proving deposition of P ions on the material surface (Fig. 8). This is due to the affinity of chitosan in the copolymer as cationic polymer to phosphate ions [24]. Therefore, the presence of the copolymer containing β-TCP filler enhance and promote the deposition of phosphorus ions which is in the favor of apatite layer formation onto its surface.

3.6.2. Characterization of the composites

The composites were investigated pre-and post-immersion for 21 days after their withdrawal from SBF by FT-IR and SEM

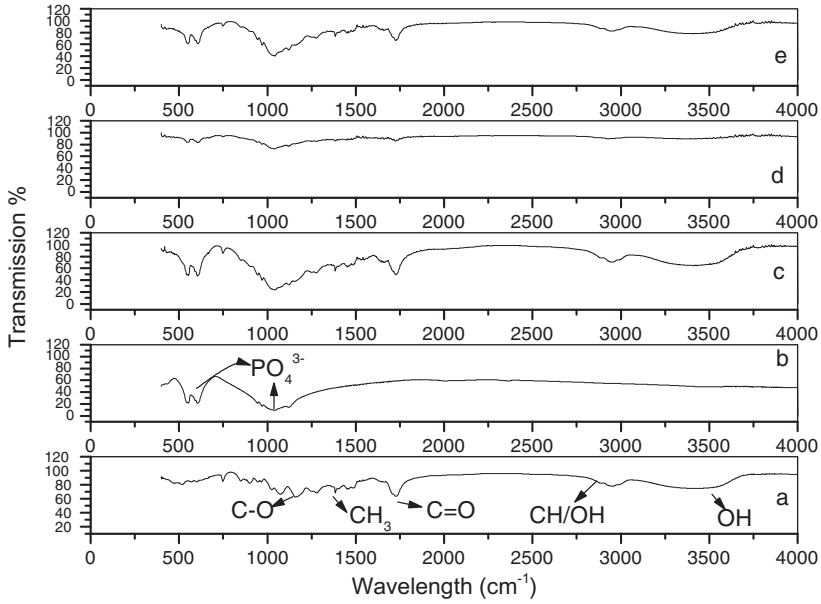

Figure 5 FT-IR of (a) copolymer, (b) β-TCP powder, (c) β-TCP/, (d) β-TCP-Z10/ and (e) β-TCP-Z20/polymeric composites.

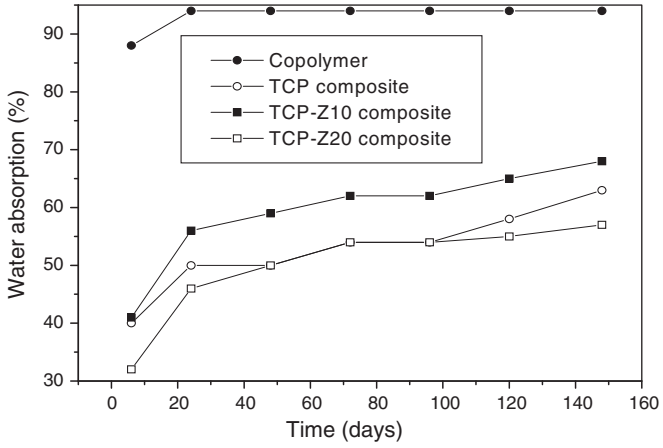

Figure 6 Water absorption % of the copolymer and the prepared composites.

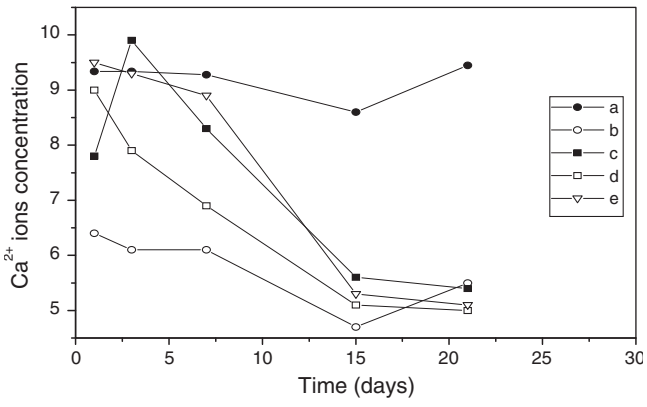

Figure 7 Concentration of Ca^{++} ions in SBF of (a) SBF (control), (b) copolymer/, (c) β-TCP/, (d) β-TCP-Z10/ and (e) β-TCP-Z20/polymeric composites.

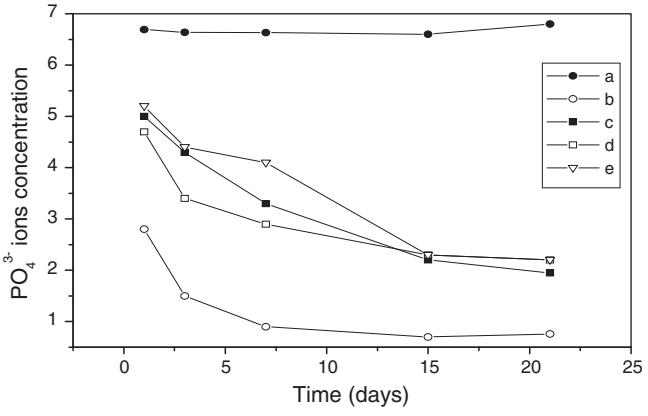

Figure 8 Concentration of phosphorus ions in SBF of (a) SBF(control), (b) copolymer, (c) β-TCP/, (d) β-TCP-Z10/ and (e) β-TCP-Z20/polymeric composites.

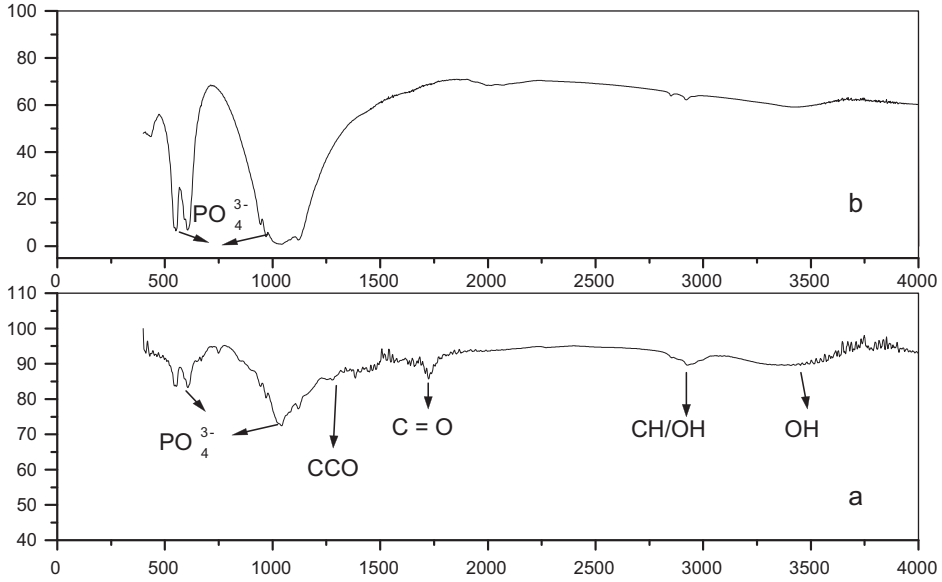

Figure 9A FT-IR of β-TCP-Z10/polymeric composite (a) pre and (b)post-immersion in SBF for 21 days.

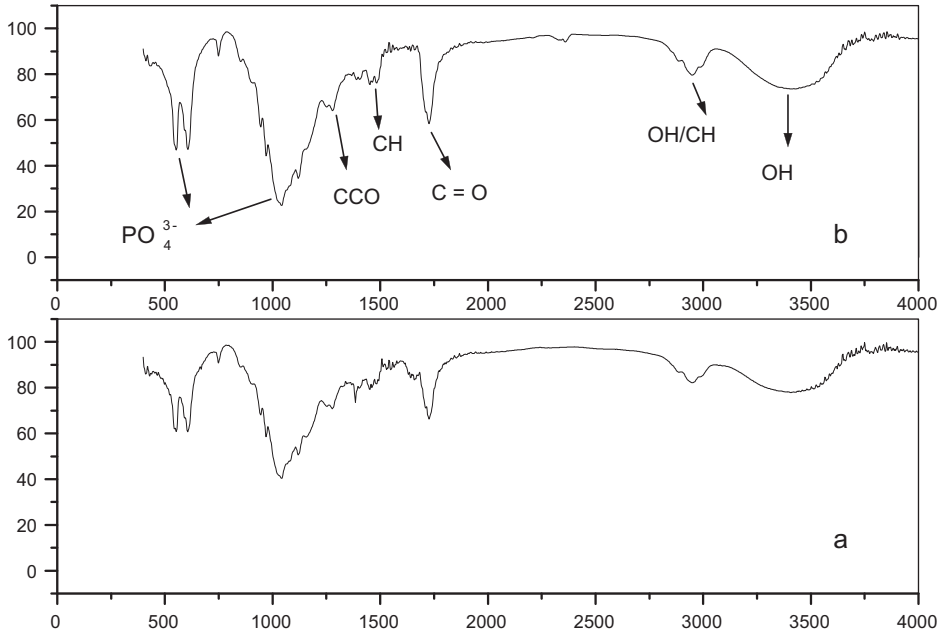

Figure 9B FT-IR of TCP-ZZ0/polymeric composite pre- and post-immersion in SBF for 21 days.

micrographs to confirm the formation of apatite layer on their surfaces.

3.6.2.1. FT-IR assessment. The optical density (O.D.) of phosphate and OH bands for β-TCP-Z10/ and β-TCP-Z20/polymeric composites were highly enhanced post-immersion compared to pre-immersion (Figs. 9A and 9B). This result proves that the presence of zirconia with β-TCP has increased the apatite formation as a result of accumulation of Zr—OH groups on the composite surface [10]. On the contrary, some of the polymer bands, such as OH, C—H, C═O, amide, CH, CH_3 and CCO disappeared post-immersion compared with the pre-immersion of β-TCP-Z10/polymeric composite denoting either masking of these bands or degradation of the polymer in SBF (Fig. 9A). The O.D. of the same polymer bands were enhanced post-immersion compared to pre-immersion for β-TCP-Z20 polymeric composite proving either the release of deposited ions into the media from the surface or reduction in polymer degradation (Figs. 9A and 9B).

3.6.2.2. Surface morphology. Fig. 10 shows SEM of the copolymer, β-TCP-Z10/ and β-TCP-Z20/polymeric composites pre- and post-immersion. Fig. 10a indicates the presence of smooth and rough surfaces with bright color proving homogeneity and chemical interaction between the two polymers and HEMA. For post-immersion, Fig. 10b shows concentrated,

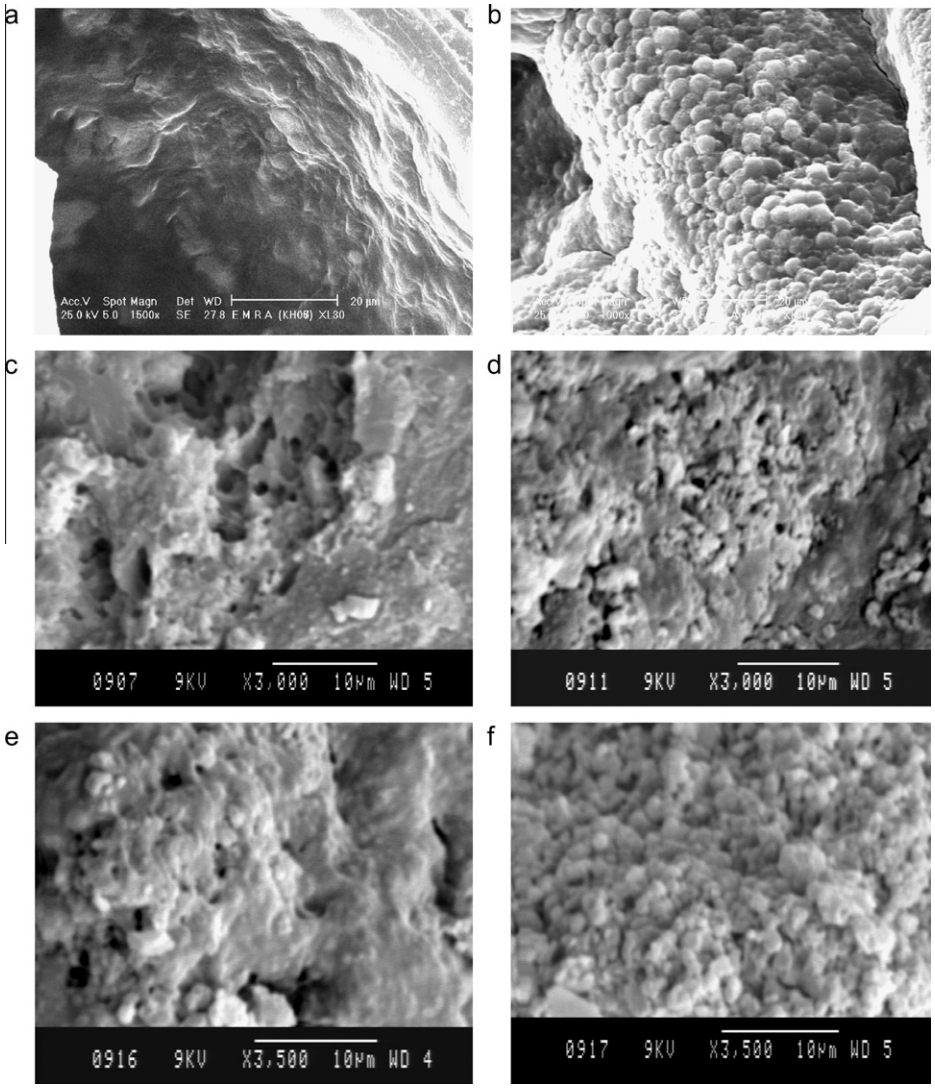

Figure 10 SEM of (a and b) copolymer, (c and d) β-TCP-Z10 composite, (e and f) β-TCP-Z20 composite of pre-and post-immersion in SBF for 21 days.

accumulated spherical particles with bright color and some pores denoting formation and nucleation of apatite onto the surface.

For β-TCP-Z10/polymeric composite, pre-immersion, Fig. 10c indicates a rough and smooth surface with many pores which are an important factor for bone growth, while post-immersion, Fig. 10d shows an increase in the number of pores with some bright particles onto the surface compared to the pre-immersion indicating the presence of zirconia particles onto the composite which react with water molecules forming Zr—OH leading to apatite formation. For β-TCP-Z20/polymeric composite, pre-immersion, Fig. 10e shows a smooth surface with some pores proving integration. While post-immersion, SEM reveals accumulated spherical particles with some minute pores on the composite surface compared to β-TCP-Z10/polymeric composites proving high content of zirconia (Fig. 10f).

4. Conclusion

The results confirmed the conversion of HA to β-TCP at 1150 °C. The FT-IR, XRD and TGA analyzes proved the presence of attached polymer onto the particles surface as in the case of β-TCP/polymeric composites containing 10% or 20% zirconia. Water absorption confirmed the stability of the composites in the media compared to the copolymer. The *in vitro* results verified that β-TCP-Z20/polymeric composite had a high ability for forming bone-like apatite layer onto its surface due to the formation of Zr—OH. The results also confirmed the role of zirconia content in the formation of apatite layer on their composite surfaces. The fabricated biocomposites are promising for bio-applications, such as bone grafting and bone tissue engineering.

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